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(30)Priority

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(54) ERYTHROPOIETIN-DEPENDENT HUMAN CELL LINE

(57)Abstract:

PURPOSE: To obtain a human cell line subject to proliferation specifically dependently on erythropoietin, thus useful for e.g. erythropoietin activity screening operations to test its cell proliferation or differentiating induction by addition of a compound which may have erythropoietin activity.

CONSTITUTION: Bone marrow fluid is taken from patient(s) with initial erythroblast leukemia, the myeloblast in the bone marrow fluid is cultured in an erythropoietin-contg. medium, and the resultant cell line formed a colony is taken, and then further cultured in a medium containing both erythropoietin and bovine fetus serum, thus obtaining the objective new human cell line AS-E2 (FIRM BP-4819) capable of subculture and subject to proliferation specifically dependently on erythropoietin. This cell line can be subjected to erythropoietin activity in vitro screening to test its cell proliferation or differentiating induction by its culture in a medium containing a compound supposed to have erythropoietin activity.

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CLAIMS

[Claim(s)]

[Claim 1] The increased human cell stock which depends for on erythropoietin specifically.

[Claim 2] The cell strain according to claim 1 which is an initial erythroblast leukemia patient's bone marrow cell origin.

[Claim 3] The cell strain according to claim 1 which is AS-E2 (the National Institute of Bioscience and Human-Technology patent microorganism deposition pin center,large, trust number FIRM:BP - 4819).

[Claim 4] The in vitro screening procedure of the erythropoietin activity which consists of adding the compound imagined to have erythropoietin activity to a cell strain according to claim 1, and examining cell proliferation or differentiation inducing.

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DETAILED DESCRIPTION

[Detailed Description of the Invention]

[0001]

[Industrial Application] This invention relates to the screening procedure of erythropoietin activity using the increased human cell stock which depends for specifically [it is still more detailed and] to erythropoietin, and this cell strain about an erythropoietin dependency human cell stock.

[0002]

[Description of the Prior Art] Erythropoietin (it may be hereafter written as Epo) is a glycoprotein which controls cell proliferation differentiation of the erythrocyte in a higher animal (for example, Carnot et al., Compt.Rend.143:384, 1986 reference). Although an erythrocyte is produced by maturation and differentiation of erythroblast in bone marrow, erythropoietin is cytokine which acts on an undifferentiated precursor cell and guides the differentiation to the erythrocyte. From such an operation, erythropoietin is greatly used as a clinical treatment medicine of ischemia, especially renal anemia in recent years.

[0003] Most erythropoietin currently used in the current clinical field is the various recombinant obtained by gene modification technology. Moreover, in recently, it is not recombination protein itself [such]. With low-molecular, more erythropoietin activity To replace with the compound which it has is tried (). [Lutz] B.Giebel and Orally Active Cytokines:Designing a Small Molecule Mimetic of Erythropoietin, IBC Conference: New Advances in Peptidemimetics & Small Molecule Design, March 23-25, 1994. In such a case, in order to present application in a clinical field with possible recombinant or low molecular weight compound, it is required to screen such erythropoietin activity simply and certainly. If it is the increased cell which depends for on erythropoietin specifically, it can be used for such a purpose.

[0004] Establishment of the human cell stock increased depending on erythropoietin is reported (Kitamura et al., J.Cell.Physiol.140:323, 1989). However, it is not specifically dependent only on erythropoietin and the cell strain named this TF-1 is a dependency also GM-CSF (granulocyte macrophage colony-stimulating factor) and IL-3 (interleukin 3).

[0005] Moreover, it is a dependency GM-CSF and IL-3 as well as [Homo sapiens megakaryoblastic leukemia cell strain UT-7 (Komatsu et al., Cancer Res.51:341, 1991) which can be maintained under existence of erythropoietin] TF-1 cell. On the other hand, establishment of the UT-7/Epo cell which disappeared GM-CSF and IL-3 dependency is reported (Komatsu et al., Blood 82:456, 1993). However, if GM-CSF of a UT-7/Epo cell and disappearance of IL-3 dependency are the things resulting from quantitative reduction of the acceptor beta chain which are GM-CSF and signal transfer protein common to IL-3 at all (Komatsu et al., above), possibility of having the SCF dependency (Auffray et al., Exp.Hematol.22:417, 1994) of UT-7 cell which is an old stock cannot be denied.

[0006] moreover, recent years and erythropoietin -- the elucidation of a specific signal transfer device is performed energetically, and many matter in connection with it is identified. However,

these researches are done using the cell (for example, Showers et al., Blood 80:3070, 1992) which made TF-1 cell (Hanazono et al., Int.J.Hematol.59:80, 1994) mentioned above and Ba/F3 cell which is a B cell system discover an erythropoietin acceptor artificially, and these cells show a dependency to not only erythropoietin but GM-CSF and IL-3. When the cell reacted to such various cytokine is used, even if it is the case where it stimulates by erythropoietin, possibility of having diverted the signal transfer path of other cytokine cannot be denied.

[0007] Therefore, it was established under erythropoietin existence, and even if it piled up the passage, the cell strain which increases only depending on erythropoietin and can be used also for discovery of signal transfer protein / gene specific to erythropoietin was not obtained.

[0008]

[Problem(s) to be Solved by the Invention] Establishment of the cell strain which can leave the property of the erythroblast which is a physiological erythropoietin target cell, and can depend for only on erythropoietin specifically, can increase in such a situation, and can use for screening of erythropoietin activity, and can be used also for discovery of signal transfer protein / gene specific to erythropoietin is called for.

[0009] The purpose of this invention is to offer the erythropoietin dependency cell strain which can be used for screening of erythropoietin activity.

[0010] This invention also makes it the purpose to offer further the in vitro screening procedure which measures erythropoietin activity using this cell strain.

[0011]

[Means for Solving the Problem] As a result of inquiring wholeheartedly that the above-mentioned purpose should be attained, out of the cell which the erythroblast which is the physiological target cell of erythropoietin leukemia-ized, this invention persons succeeded in establishing the starting cell strain, and completed this invention.

[0012] That is, this invention offers the increased human cell stock which depends for on erythropoietin specifically.

[0013] Furthermore, the in vitro screening procedure of the erythropoietin activity which consists of adding the compound guessed that this invention has erythropoietin activity to the above-mentioned cell strain, and examining cell proliferation or differentiation inducing is offered.

[0014] The cell strain of this invention can be established using an extraction **** cell from for example, an initial erythroblast leukemia (early erthroblastic leukemia) patient's bone marrow etc. Especially in this invention, the myeloblast of the patient by whom the blast cell was accepted in bone marrow 76.2% was extracted. This cell formed the colony by Epo addition. When this blast cell was furthermore cultivated in FCS (fetal calf serum) and IMDM (chair cove alteration Dulbecco culture medium) which carried out Epo addition, the passage was able to become possible, and the stable cell strain was able to be established. Thus, it is named AS-E2 and the established cell strain is deposited with the National Institute of Bioscience and Human-Technology patent microorganism deposition pin center, large by trust number FIRM:BP -4819 (October 5, 1994 deposition).

[0015] The par.oxodase reaction (PO) is negative and AS-E2 accepted megaloblast Mr. change (periodic acid-Schiff reagent-AS positivity).

[0016] Moreover, when the surface antigen of cell strain AS-E2 was examined, CD71, CD36, glycophorins A (GPA), CD38, and CD19, etc. were discovered.

[0017] When the reactivity over the various cytokine of AS-E2 was examined, only when it incubated with Epo, it was stimulated notably, and it was shown that AS-E2 reacts to Epo specifically, and increases. Furthermore, in order to examine the growth inhibition by the reactivity of Epo to AS-E2, and the anti-Epo antibody, when the number of cells was counted under the microscope and the viable count was counted by the trypan blue, it became clear that cell proliferation remarkable only in Epo addition conditions is accepted, and such growth effectiveness is controlled by addition of an anti-Epo antibody.

[0018] Moreover, the manifestation of the Epo acceptor in AS-E2 was checked from the joint

experiment of Radiolabel Epo. As a result of analyzing the property of an Epo acceptor using Scatchard analysis from the data furthermore obtained, the Epo acceptor which AS-E2 discovers was only a low compatibility mold.

[0019] Recently, Epo combines with an Epo acceptor, phosphorylation of Jak2 which is one sort of Jak family tyrosine kinase is carried out by this, and the transfer path of the growth signal that Jak2 self phosphorylates the following molecule with kinase activity is further advocated by this phosphorylation (Ihle et al., TIBS 19:222, 1994). In order to examine the justification of this assumption, after cultivating AS-E2 under Epo nonexistence for 24 hours and adding Epo subsequently, the cell was solubilized and immunoprecipitation was performed by anti-Jak2 antibody or the anti-Epo acceptor antibody. After giving sediment to SDS-PAGE, as a result of imprinting on the PVDF film and detecting tyrosin phosphorylation protein by the anti-phosphorylated tyrosine antibody, AS-E2 reacted to Epo, and the Epo acceptor and the tyrosin phosphorylation of Jak2 which are an Epo signal transfer system were accepted. This can call it the result to suggest that the above-mentioned growth signal transfer path is right.

[0020] the erythrocyte sent from the same acceptor apart from a growth signal -- although existence of the signal of specific cell differentiation is suggested -- this -- current -- it is not solved most. however, the erythrocyte from an Epo acceptor -- the erythrocyte of the lower stream of a river which the signal which activates a series of specific transcription factor groups is transmitted, and is controlled by these transcription factors -- the imprint of a specific gene cluster (a heme synthetase gene and globin gene) is activated, and it is thought that it finally specializes in a hemoglobin production cell (Yamamoto M. the experimental medicine 11:1026 and 1993). the place which examined the manifestation of mRNA of GATA-1 and GATA-2 which is this specific.. transcription factor by the Northern blot -- AS-E2 -- both GATA-1 and GATA-2 -- although -- it was checked that it is discovered.

[0021] It was shown that it is the cell strain for which AS-E2 depends only on Epo specifically and which it increases from the various descriptions of such AS-E2.

[0022] Therefore, the cell strain of this invention is useful although Epo activity, such as a low molecular weight compound imagined to have the various recombinant Epo or Epo activity, is screened by in vitro one simply and certainly. Such screening can be carried out by examining cell proliferation or differentiation inducing. For example, the incubation of the compound imagined to have Epo activity and the cell strain of this invention is mixed and carried out. The cell proliferation after fixed time amount The incorporation trial of 3T-thymidine, Or [whether it measures by the MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide) assay method (J.Immun.Methods 65:55, 1983 reference), and] Or it can carry out by measuring a viable count etc. Moreover, it is also possible to measure Epo concentration by this examining method. Furthermore, the cell strain of this invention can be used as the symptoms analysis and the therapy model of initial erythroblast leukemia. Furthermore, as mentioned above, the cell strain of this invention can be used also for the experiment for solving specific signal transfer protein / gene to erythropoietin.

[0023] Although an example explains this invention in more detail below, the range of this invention is not limited to this.

[0024]

[Example]

Example 1: Bone marrow liquid was extracted from establishment of AS-E2, and the initial erythroblast leukemia patient of maintenance. As for this patient, the blast cell [light microscope PO (-) PAS(-) CD11b(-) CD13(+) CD33(+) CD10(-) CD19(-) glycophorin A(+) chromosome:46, XY] was accepted in bone marrow 76.2% at the time of the first medical examination. This patient's myeloblast formed the colony by Epo addition. Subsequently, it is this blast cell Epo (2u/ml) and 20% It was stabilized when cultivated in the FCS addition IMDM, and the passage became possible, and it was established as a cell strain. This cell strain was named AS-E2.

[0025] The gestalt and surface antigen of AS-E2 are as follows.

When gestalt AS-E2 of AS-E2 is observed under a microscope, it is about 30micro of ****, and has the vacuole. PO was negative and PAS was a positivity.

The fluorescent antibody technique (Fried et al., Flow Cytometry, Boca Raton, CRC Press:59-78, 1989) by flow cytometer considered the manifestation of the surface antigen of cell strain AS-E2 established by analysis this example of the surface antigen of AS-E2 using the panel of the monoclonal antibody to various Homo sapiens antigens.

[0026] The obtained result is shown in the following table 1.

[0027]

table 1 [] The rate of a positivity (%) CD2 0.2 CD10 0.2 CD19 6.5 CD25 0 CD34 0.7 CD36 98.8 CD38 14.1 CD41 0.7CDs71 99.1 HLA-DR 1.1 glycophorin A AS-E2 had discovered CD71, CD36, glycophorins A (GPA), CD38, and CD19, etc. so that clearly from 43.1 tables.

[0028] Example 2: The reactivity to the various cytokine of cell strain AS-E2 established in the reactant example 1 over various cytokine was examined.

[0029] AS-E2 Various cytokine is added to 5,000 cells / well, and it is 5%. CO₂, 95% It cultivated for three days at air (100% of humidity), and 37 degrees C. The class of used cytokine The following passes along concentration and it comes out. And a certain :human erythropoietin : 10u/ml Homo sapiens interleukin 1 alpha (hEPO) :10 ng/ml Homo sapiens interleukin 1 beta (hIL-1alpha) :10 ng/ml Homo sapiens interleukin 2 (hIL-1beta) :10 ng/ml Homo sapiens interleukin 3 (hIL-2) :100 ng/ml Homo sapiens interleukin 4 (hIL-3) :10 ng/ml Homo sapiens interleukin 6 (hIL-4) :10 ng/ml Homo sapiens interleukin 7 (hIL-6) :10 ng/ml Homo sapiens interleukin 8 (hIL-7) (hIL-8) :1000 ng/ml Homo sapiens 11 (hIL-11):10 ng/ml interleukin Homo sapiens granulocyte colony-stimulating factor (hG-CSF): -- 100u/ml Homo sapiens granulocyte macrophage colony-stimulating factor (hGM-CSF): -- 10 ng/ml The Homo sapiens leukemia inhibitor : 10 ng/ml human erythrocyte differentiator (hLIF) : 10 ng/ml Homo sapiens stem cell factor (hEDF) : (hSCF) 10 ng(s)/ml Homo sapiens transforming growth factor - beta : (HTGF-beta) 100 ng(s)/ml Homo sapiens basicity fibroblast growth factor (hbFGF) :10 ng/ml Homo sapiens hepatocyte growth factor (hHGF): -- 500 ng/ml Homo sapiens M:100 ng/ml oncostatin Homo sapiens epidermal growth factor (hEGF): -- after 1000 ng/ml culture -- CellTiter96TM AQueous Non-Radioactive Cell Proriferation Assay The number of cells was measured by measuring the absorbance in 490nm using Kit (product made from Promega). The blank which does not contain a cell, and the contrast which does not contain cytokine were examined to coincidence. The obtained result is shown in drawing 1 . Only when it incubated with Epo, growth was stimulated notably, and AS-E2 was not stimulated depending on other cytokine so that clearly from drawing.

Example 3: The Epo reactivity (growth activity) of cell strain AS-E2 established in the growth inhibition example 1 by Epo reactivity and the anti-Epo antibody and the growth activity by the anti-Epo antibody were examined.

[0030] without it adds (1) Epo using 1xAS-E2 cell 105 cells / ml -- culture and (2) Epo 2u/ml -- adding -- culture and (3) Epo 2u/ml and an anti-Epo antibody (it is diluted and used for alphaEpo:1/100) -- adding -- cultivating -- being with time (2, 4, 6, and 8 days) -- the number of cells was counted under the microscope. The obtained result is shown in drawing 2 . As compared with the cell in which the cell which carried out Epo addition does not carry out Epo addition, the remarkable increment in the number of cells was observed so that clearly from drawing. Moreover, when an anti-Epo antibody was added to coincidence, this growth activity was checked.

[0031] Subsequently, the result of having dyed the cell of the three above-mentioned group by the trypan blue, and having counted the viable count is shown in drawing 3 . Although the viable count was decreasing with time in the cell which does not carry out Epo addition, and the cell which added Epo and an anti-Epo antibody so that clearly from drawing, most which was counted as the number of cells counted also as a viable count in the cell which carried out Epo addition.

Example 4: It checked by the following approaches that the Epo acceptor was discovered on the check AS-E2 cell of a manifestation of an Epo acceptor.

[0032] AS-E2 cell was cultivated under 24-hour Epo nonexistence, 0.06-3nM addition of 125 I-Epo (product made from Amersham) was carried out to 1×10^6 cells, and joint assay was performed on 15 degrees C and the conditions of 3 hours. Nonspecific association was measured under the non-indicator Epo existence of the amount of 400 times of 125 I-Epo. In addition, the presentation of a joint assay culture medium is 2%. BSA, 0.2% It is an IMDM culture medium containing NaN₃. Obtained association is shown in drawing 4. From this, it was checked that the Epo acceptor is discovered on AS-E2 cell.

[0033] Subsequently, when Scatchard analysis was performed from the data obtained from joint assay and having been asked for the dissociation constant (K_d value) and the number of acceptors of an Epo acceptor, it was shown that the Epo acceptor which AS-E2 cell has discovered is one kind of low compatibility mold.

Example 5: The phosphorylation of the Jak2 and the Epo acceptor in Jak2 and phosphorylation AS-E2 of an Epo acceptor was examined by the following approaches, and the signal transfer path of Epo was examined.

[0034] After having cultivated AS-E2 under Epo nonexistence for 24 hours, adding Epo (0 or 10u/ml) subsequently and making it react for 5 minutes, 1% NP-40 (Nakarai Tesuku, Inc. make), 10% Glycerol, 1mM PMSF (the Wako Pure Chem Industries make) and 0.1mg/ml Leupeptin (the Wako Pure Chem Industries make), 5microM pepstatin A (the Wako Pure Chem Industries make), 1mM EDTA, 2mM The cell was solubilized with the tris buffer salting in liquid containing alt.banazin san sodium (SIGMA company make). Immunoprecipitation was performed using this by anti-Jak2 antibody or the anti-Epo acceptor antibody (all are UPSTATE BIOTECHNOLOGY and INC. (USA) company make). After giving sediment to SDS-PAGE, it imprinted on the PVDF film and thyrosin phosphorylation protein was detected by the anti-phosphorylated tyrosine antibody (Wako Pure Chem, Inc. make). the obtained result is shown in drawing 5 (A:anti-Jak2 antibody, a B:anti-Epo acceptor antibody, and A and B -- also in any, a lane 1 expresses the case where a lane 2 carries out Epo addition of the Epo case of not adding). AS-E2 reacted to Epo, and the thyrosin phosphorylation of Jak2 which is an Epo signal transfer system, and an Epo acceptor was accepted so that clearly from drawing.

Example 6: The following Northern blot analysis considered the manifestation of mRNA of GATA-1, GATA-1 in manifestation AS-E2 of mRNA of GATA-2, and GATA-2.

[0035] All RNA was prepared according to the formula of kit attachment from K562 cell strain (chronic-myelogenous-leukemia acute transformation cell strain) and HL-60 cell strain (acute leukemia cell strain), using ISOGEN (NIPPON GENE make) as cell strain AS-E2 and contrast of this invention. Subsequently, Northern blot analysis (Molecular Cloning: A Laboratory Manual, Sambrook et al., Cold Spring Harbor Laboratory Press, 1989) was performed. The indicator of a probe is Random. Primer It carried out using LabellingKit (product made from Takara). Namely, Eco of a PVC vector GATA-1 (Zon et al., Proc.Natl.Acad.Sci.USA 88:10638-10641, 1991) inserted in RI part and the insertion which codes GATA-2 (Nagai et al., Blood 84:1074-1084, 1994) were started, and the indicator probe was produced according to the formula of kit attachment:

[0036] After doing 30microg use per one lane of all RNA from each above-mentioned cell and performing agarose gel electrophoresis, Hybond-N (product made from Amersham) was used as a hybridization transfer membrane, and blotting was carried out by the capillary tube method. Hybridization is 50%. A formamide, 5xSSPE, 2xDenhardt's solution, 0.1% It carried out at 42 degrees C overnight using the solution which consists of a presentation of SDS. They are after hybridization and a membrane 2xSSC and 0.1% The room temperature washed 4 times by SDS, autoradiography was further performed in BAS (product made from Fuji), and image analysis was carried out. the result is shown in drawing 6 (A:GATA -1, B:GATA -2, and A and B -- all are boiled and, in AS-E2, a lane 2K562, and a lane 3, a lane 1 expresses HL-60). GATA-1 was detected by AS-E 2 andK562 as a clear band, and GATA-2 were accepted by any cell strain of AS-E2, K562, and HL-60 so that clearly from drawing.

[0037]

[Effect of the Invention] By this invention, it was specifically dependent only on erythropoietin, and increased, and the establishment of a cell strain which can be used for screening of erythropoietin activity was attained. The in vitro screening procedure which measures the erythropoietin activity of the compound presumed to have erythropoietin activity using the cell strain of this invention became possible. This in vitro screening can be carried out by examining the cell proliferation or differentiation inducing of a cell strain of this invention. Moreover, it is also possible to measure Epo concentration by this examining method. Furthermore, the cell strain of this invention can be used as the symptoms analysis and the therapy model of initial erythroblast leukemia. Furthermore, the experiment for solving specific signal transfer protein / gene to erythropoietin can be conducted using the cell strain of this invention.

[0038] The utility value of the erythropoietin dependency human cell stock of these points to this invention is very large.

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TECHNICAL FIELD

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PRIOR ART

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EFFECT OF THE INVENTION

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MEANS

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[0012] That is, this invention offers the increased human cell stock which depends for on erythropoietin specifically.

[0013] Furthermore, the in vitro screening procedure of the erythropoietin activity which consists of adding the compound guessed that this invention has erythropoietin activity to the above-mentioned cell strain, and examining cell proliferation or differentiation inducing is offered.

[0014] The cell strain of this invention can be established using an extraction **** cell from for example, an initial erythroblast leukemia (early erhthroblastic leukemia) patient's bone marrow etc. Especially in this invention, the myeloblast of the patient by whom the blast cell was accepted in bone marrow 76.2% was extracted. This cell formed the colony by Epo addition. When this blast cell was furthermore cultivated in FCS (fetal calf serum) and IMDM (chair cove alteration Dulbecco culture medium) which carried out Epo addition, the passage was able to become possible, and the stable cell strain was able to be established. Thus, it is named AS-E2 and the established cell strain is deposited with the National Institute of Bioscience and Human-Technology patent microorganism deposition pin center,large by trust number FIRM:BP -4819 (October 5, 1994 deposition).

[0015] The par pxodase reaction (PO) is negative and AS-E2 accepted megaloblast Mr. change (periodic acid-Schiff reagent-AS positivity).

[0016] Moreover, when the surface antigen of cell strain AS-E2 was examined, CD71, CD36, glycophorins A (GPA), CD38, and CD19, etc. were discovered.

[0017] When the reactivity over the various cytokine of AS-E2 was examined, only when it incubated with Epo, it was stimulated notably, and it was shown that AS-E2 reacts to Epo specifically, and increases. Furthermore, in order to examine the growth inhibition by the reactivity of Epo to AS-E2, and the anti-Epo antibody, when the number of cells was counted under the microscope and the viable count was counted by the trypan blue, it became clear that cell proliferation remarkable only in Epo addition conditions is accepted, and such growth effectiveness is controlled by addition of an anti-Epo antibody.

[0018] Moreover, the manifestation of the Epo acceptor in AS-E2 was checked from the joint experiment of Radiolabel Epo. As a result of analyzing the property of an Epo acceptor using Scatchard analysis from the data furthermore obtained, the Epo acceptor which AS-E2 discovers was only a low compatibility mold.

[0019] Recently, Epo combines with an Epo acceptor, phosphorylation of Jak2 which is one sort of Jak family tyrosine kinase is carried out by this, and the transfer path of the growth signal that Jak2 self phosphorylates the following molecule with kinase activity is further advocated by this phosphorylation (Ihle et al., TIBS 19:222, 1994). In order to examine the justification of this

assumption, after cultivating AS-E2 under Epo nonexistence for 24 hours and adding Epo subsequently, the cell was solubilized and immunoprecipitation was performed by anti-Jak2 antibody or the anti-Epo acceptor antibody. After giving sediment to SDS-PAGE, as a result of imprinting on the PVDF film and detecting thyrosin phosphorylation protein by the anti-phosphorylated tyrosine antibody, AS-E2 reacted to Epo, and the Epo acceptor and the thyrosin phosphorylation of Jak2 which are an Epo signal transfer system were accepted. This can call it the result to suggest that the above-mentioned growth signal transfer path is right.

[0020] the erythrocyte sent from the same acceptor apart from a growth signal -- although existence of the signal of specific cell differentiation is suggested -- this -- current -- it is not solved most. however, the erythrocyte from an Epo acceptor -- the erythrocyte of the lower stream of a river which the signal which activates a series of specific transcription factor groups is transmitted, and is controlled by these transcription factors -- the imprint of a specific gene cluster (a heme synthetase gene and globin gene) is activated, and it is thought that it finally specializes in a hemoglobin production cell (Yamamoto M. the experimental medicine 11:1026 and 1993). the place which examined the manifestation of mRNA of GATA-1 and GATA-2 which is this specific transcription factor by the Northern blot -- AS-E2 -- both GATA-1 and GATA-2 -- although -- it was checked that it is discovered.

[0021] It was shown that it is the cell strain for which AS-E2 depends only on Epo specifically and which it increases from the various descriptions of such AS-E2.

[0022] Therefore, the cell strain of this invention is useful although Epo activity, such as a low molecular weight compound imagined to have the various recombinant Epo or Epo activity, is screened by in vitro one simply and certainly. Such screening can be carried out by examining cell proliferation or differentiation inducing. For example, the incubation of the compound imagined to have Epo activity and the cell strain of this invention is mixed and carried out. The cell proliferation after fixed time amount The incorporation trial of 3T-thymidine, Or [whether it measures by the MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide) assay method (J.Immun.Methods 65:55, 1983 reference), and] Or it can carry out by measuring a viable count etc. Moreover, it is also possible to measure Epo concentration by this examining method. Furthermore, the cell strain of this invention can be used as the symptoms analysis and the therapy model of initial erythroblast leukemia. Furthermore, as mentioned above, the cell strain of this invention can be used also for the experiment for solving specific signal transfer protein / gene to erythropoietin.

[0023] Although an example explains this invention in more detail below, the range of this invention is not limited to this.

[Translation done.]

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EXAMPLE

[Example]

Example 1: Bone marrow liquid was extracted from establishment of AS-E2, and the initial erythroblast leukemia patient of maintenance. As for this patient, the blast cell [light microscope PO (-) PAS(-) CD11b(-) CD13(+) CD33(+) CD10(-) CD19(-) glycoporphin A(+) chromosome:46, XY] was accepted in bone marrow 76.2% at the time of the first medical examination. This patient's myeloblast formed the colony by Epo addition. Subsequently, it is this blast cell Epo (2u/ml) and 20% It was stabilized when cultivated in the FCS addition IMDM, and the passage became possible, and it was established as a cell strain. This cell strain was named AS-E2.

[0025] The gestalt and surface antigen of AS-E2 are as follows.

When gestalt AS-E2 of AS-E2 is observed under a microscope, it is about 30micro of ****, and has the vacuole. PO was negative and PAS was a positivity.

The fluorescent antibody technique (Fried et al., Flow Cytometry, Boca Raton, CRC Press:59-78, 1989) by flow cytometer considered the manifestation of the surface antigen of cell strain AS-E2 established by analysis this example of the surface antigen of AS-E2 using the panel of the monoclonal antibody to various Homo sapiens antigens.

[0026] The obtained result is shown in the following table 1.

[0027]

table 1 [] The rate of a positivity (%) CD2 0.2 CD10 0.2 CD19 6.5 CD25 0 CD34 0.7 CD36 98.8 CD38 14.1 CD41 0.7CDs71 99.1 HLA-DR 1.1 glycoporphin A AS-E2 had discovered CD71, CD36, glycoporphins A (GPA), CD38, and CD19, etc. so that clearly from 43.1 tables.

[0028] Example 2: The reactivity to the various cytokine of cell strain AS-E2 established in the reactant example 1 over various cytokine was examined.

[0029] AS-E2 Various cytokine is added to 5,000 cells / well, and it is 5%. CO2, 95% It cultivated for three days at air (100% of humidity), and 37 degrees C. The class of used cytokine The following passes along concentration and it comes out. And a certain :human erythropoietin : 10u/ml Homo sapiens interleukin 1 alpha (hEPO) :10 ng/ml Homo sapiens interleukin 1 beta (hIL-1alpha) :10 ng/ml Homo sapiens interleukin 2 (hIL-1beta) :10 ng/ml Homo sapiens interleukin 3 (hIL-2) :100 ng/ml Homo sapiens interleukin 4 (hIL-3) :10 ng/ml Homo sapiens interleukin 6 (hIL-4) :10 ng/ml Homo sapiens interleukin 7 (hIL-6) :10 ng/ml Homo sapiens interleukin 8 (hIL-7) (hIL-8) :1000 ng/ml Homo sapiens 11 (hIL-11):10 ng/ml interleukin Homo sapiens granulocyte colony-stimulating factor (hG-CSF): -- 100u/ml Homo sapiens granulocyte macrophage colony-stimulating factor (hGM-CSF): -- 10 ng/ml The Homo sapiens leukemia inhibitor : 10 ng/ml human erythrocyte differentiator (hLIF) : 10 ng/ml Homo sapiens stem cell factor (hEDF) : (hSCF) 10 ng(s)/ml Homo sapiens transforming growth factor - beta : (HTGF-beta) 100 ng(s)/ml Homo sapiens basicity fibroblast growth factor (hbFGF) :10 ng/ml Homo sapiens hepatocyte growth factor (hHGF): -- 500 ng/ml Homo sapiens M:100 ng/ml oncostatin Homo sapiens epidermal growth factor (hEGF): -- after 1000 ng/ml culture -- CellTiter96TM AQueous Non-Radioactive Cell Proriferation Assay The number of cells was

measured by measuring the absorbance in 490nm using Kit (product made from Promega). The blank which does not contain a cell, and the contrast which does not contain cytokine were examined to coincidence. The obtained result is shown in drawing 1. Only when it incubated with Epo, growth was stimulated notably, and AS-E2 was not stimulated depending on other cytokine so that clearly from drawing.

Example 3: The Epo reactivity (growth activity) of cell strain AS-E2 established in the growth inhibition example 1 by Epo reactivity and the anti-Epo antibody and the growth activity by the anti-Epo antibody were examined.

[0030] without it adds (1) Epo using 1xAS-E2 cell 105 cells / ml -- culture and (2) Epo 2u/ml -- adding -- culture and (3) Epo 2u/ml and an anti-Epo antibody (it is diluted and used for alphaEpo:1/100) -- adding -- cultivating -- being with time (2, 4, 6, and 8 days) -- the number of cells was counted under the microscope. The obtained result is shown in drawing 2. As compared with the cell in which the cell which carried out Epo addition does not carry out Epo addition, the remarkable increment in the number of cells was observed so that clearly from drawing. Moreover, when an anti-Epo antibody was added to coincidence, this growth activity was checked.

[0031] Subsequently, the result of having dyed the cell of the three above-mentioned group by the trypan blue, and having counted the viable count is shown in drawing 3. Although the viable count was decreasing with time in the cell which does not carry out Epo addition, and the cell which added Epo and an anti-Epo antibody so that clearly from drawing, most which was counted as the number of cells counted also as a viable count in the cell which carried out Epo addition.

Example 4: It checked by the following approaches that the Epo acceptor was discovered on the check AS-E2 cell of a manifestation of an Epo acceptor.

[0032] AS-E2 cell was cultivated under 24-hour Epo nonexistence, 0.06-3nM addition of 125 I-Epo (product made from Amersham) was carried out to 1x10⁶ cells, and joint assay was performed on 15 degrees C and the conditions of 3 hours. Nonspecific association was measured under the non-indicator Epo existence of the amount of 400 times of 125 I-Epo. In addition, the presentation of a joint assay culture medium is 2%. BSA, 0.2% It is an IMDM culture medium containing NaN₃. Obtained association is shown in drawing 4. From this, it was checked that the Epo acceptor is discovered on AS-E2 cell.

[0033] Subsequently, when Scatchard analysis was performed from the data obtained from joint assay and having been asked for the dissociation constant (K_d value) and the number of acceptors of an Epo acceptor, it was shown that the Epo acceptor which AS-E2 cell has discovered is one kind of low compatibility mold.

Example 5: The phosphorylation of the Jak2 and the Epo acceptor in Jak2 and phosphorylation AS-E2 of an Epo acceptor was examined by the following approaches, and the signal transfer path of Epo was examined.

[0034] After having cultivated AS-E2 under Epo nonexistence for 24 hours, adding Epo (0 or 10u/ml) subsequently and making it react for 5 minutes, 1% NP-40 (Nakarai Tesuku, Inc. make), 10% Glycerol, 1mM PMSF (the Wako Pure Chem Industries make) and 0.1mg/ml Leupeptin (the Wako Pure Chem Industries make), 5microM pepstatin A (the Wako Pure Chem Industries make), 1mM EDTA, 2mM The cell was solubilized with the tris buffer salting in liquid containing alt.banazin san sodium (SIGMA company make). Immunoprecipitation was performed using this by anti-Jak2 antibody or the anti-Epo acceptor antibody (all are UPSTATE BIOTECHNOLOGY and INC. (USA) company make). After giving sediment to SDS-PAGE, it imprinted on the PVDF film and thyrosin phosphorylation protein was detected by the anti-phosphorylated tyrosine antibody (Wako Pure Chem, Inc. make). the obtained result is shown in drawing 5 (A:anti-Jak2 antibody, a B:anti-Epo acceptor antibody, and A and B -- also in any, a lane 1 expresses the case where a lane 2 carries out Epo addition of the Epo case of not adding). AS-E2 reacted to Epo, and the thyrosin phosphorylation of Jak2 which is an Epo signal transfer system, and an Epo acceptor was accepted so that clearly from drawing.

Example 6: The following Northern blot analysis considered the manifestation of mRNA of GATA-1, GATA-1 in manifestation AS-E2 of mRNA of GATA-2, and GATA-2.

[0035] All RNA was prepared according to the formula of kit attachment from K562 cell strain (chronic-myelogenous-leukemia acute transformation cell strain) and HL-60 cell strain (acute leukemia cell strain), using ISOGEN (NIPPON GENE make) as cell strain AS-E2 and contrast of this invention. Subsequently, Northern blot analysis (Molecular Cloning: A Laboratory Manual, Sambrook et al., Cold Spring Harbor Laboratory Press, 1989) was performed. The indicator of a probe is Random. Primer It carried out using LabellingKit (product made from Takara). Namely, Eco of a PVC vector GATA-1 (Zon et al., Proc.Natl.Acad.Sci.USA 88:10638-10641, 1991) inserted in RI part and the insertion which codes GATA-2 (Nagai et al., Blood 84:1074-1084, 1994) were started, and the indicator probe was produced according to the formula of kit attachment.

[0036] After doing 30microg use per one lane of all RNA from each above-mentioned cell and performing agarose gel electrophoresis, Hybond-N (product made from Amersham) was used as a hybridization transfer membrane, and blotting was carried out by the capillary tube method. Hybridization is 50%. A formamide, 5xSSPE, 2xDenhardt's solution, 0.1% It carried out at 42 degrees C overnight using the solution which consists of a presentation of SDS. They are after hybridization and a membrane 2xSSC and 0.1% The room temperature washed 4 times by SDS, autoradiography was further performed in BAS (product made from Fuji), and image analysis was carried out. the result is shown in drawing 6 (A:GATA -1, B:GATA -2, and A and B -- all are boiled and, in AS-E2, a lane 2K562, and a lane 3, a lane 1 expresses HL-60). GATA-1 was detected by AS-E 2 andK562 as a clear band, and GATA-2 were accepted by any cell strain of AS-E2, K562, and HL-60 so that clearly from drawing.

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DESCRIPTION OF DRAWINGS

[Brief Description of the Drawings]

[Drawing 1] It is drawing showing the reactivity of the various cytokine to AS-E2 cell.

[Drawing 2] It is drawing showing the number of cells with time observed under the microscope to show the Epo reactivity of AS-E2, and the growth activity by the anti-Epo antibody. (-) is Epo. For the cell which added 2u/ml, and (**), the cell which does not add Epo, and (**) are Epo. The cell which added 2u/ml and an anti-Epo antibody (alphaEpo:1/100 dilution) is expressed.

[Drawing 3] It is drawing showing the viable count with time which carried out trypan blue dyeing to show the Epo reactivity of AS-E2, and the growth activity by the anti-Epo antibody. (-) is Epo. The cell which added 2u/ml, the cell into which (**) does not add Epo, and (**) express the cell which added Epo2u/ml and an anti-Epo antibody (alphaEpo:1/100 dilution).

[Drawing 4] It is drawing showing the joint assay of AS-E2 using 125 I-Epo. In (O), all avidity and (**) express nonspecific avidity, and (-) expresses specific binding activity.

[Drawing 5] It is drawing (photograph of electrophoresis) showing the phosphorylation of the Jak2 and the Epo acceptor in AS-E2. A gives an immune precipitate with an anti-Epo acceptor antibody (lane 1 Epo un-adding; the lane 2 Epo addition) to anti-Jak2 antibody (lane 1 Epo un-adding; the lane 2 Epo addition) among drawing, B is given to the electrophoresis of SDS-PAGE, it imprints on the PVDF film, and drawing which detected thyrosin phosphorylation protein by the anti-phosphorylated tyrosine antibody is expressed.

[Drawing 6] It is drawing (photograph of electrophoresis) showing the manifestation of mRNA of AS-E2, K562, GATA-1 in HL-60 cell strain, and GATA-2. The result of the Northern blot for which A used GATA-1 (lane 1 AS-E2; lane 2 K562; the lane 3 HL-60) for among drawing, and B used GATA-2 (lane 1 AS-E2; lane 2 K562; the lane 3 HL-60) as a probe is expressed.

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(54)【発明の名称】 エリスロポエチン依存性ヒト細胞株

(57)【要約】

【目的】 エリスロポエチンのみに特異的に依存して増殖し、エリスロポエチン活性のスクリーニングに用い得る細胞株を樹立する。

【構成】 エリスロポエチンに特異的に依存して増殖するヒト細胞株、およびエリスロポエチン活性を有すると推察される化合物を上記細胞株に添加して細胞増殖または分化誘導を試験することからなるエリスロポエチン活性のインビトロスクリーニング法。

【特許請求の範囲】

【請求項1】 エリスロポエチンに特異的に依存して増殖するヒト細胞株。

【請求項2】 初期赤芽球白血病患者の骨髓細胞由来である請求項1に記載の細胞株。

【請求項3】 AS-E2（生命工学工業技術研究所特許微生物寄託センター、受託番号FIRM:BP-4819）である請求項1に記載の細胞株。

【請求項4】 エリスロポエチン活性を有すると推察される化合物を請求項1に記載の細胞株に添加して細胞増殖または分化誘導を試験することからなるエリスロポエチン活性のインビトロスクリーニング法。

【発明の詳細な説明】

【0001】

【産業上の利用分野】本発明はエリスロポエチン依存性ヒト細胞株に関し、さらに詳しくはエリスロポエチンに特異的に依存して増殖するヒト細胞株、および該細胞株を用いるエリスロポエチン活性のスクリーニング法に関する。

【0002】

【従来の技術】エリスロポエチン（以下、Epoと略記することもある）は高等動物における赤血球の細胞増殖分化を制御する糖タンパク質である（例えば、Carnot et al., Compt. Rend. 143:384, 1986参照）。赤血球は骨髓中で赤芽球の成熟および分化により生産されるが、エリスロポエチンは未分化の前駆細胞に作用してその赤血球への分化を誘導するサイトカインである。そのような作用から、エリスロポエチンは貧血、特に腎性貧血の臨床治療薬として近年大いに使用されている。

【0003】現在臨床分野で使用されているエリスロポエチンのほとんどは遺伝子組換え技術によって得られる各種組換え体である。また、最近ではこのような組換えタンパク質そのものではなく、もっと低分子でエリスロポエチン活性をもつ化合物で置き換えることが試みられている（Lutz B. Giebel, Orally Active Cytokines: Designing a Small Molecule Mimetic of Erythropoietin, IBC Conference: New Advances in Peptidomimetics & Small Molecule Design, March 23-25, 1994）。このような場合、可能性のある組換え体または低分子化合物を臨床分野での応用に供するためには、これらのエリスロポエチン活性を簡単かつ確実にスクリーニングすることが必要である。エリスロポエチンに特異的に依存して増殖する細胞であれば、このような目的に使用することができる。

【0004】エリスロポエチンに依存して増殖するヒト細胞株の樹立が報告されている（Kitamura et al., J. Cell. Physiol. 140:323, 1989）。しかしながら、このTF-1と命名された細胞株はエリスロポエチンのみに特異的に依存するのではなく、GM-CSF（顆粒球マクロファージコロニー刺激因子）やIL-3（インター

ロイキン-3）にも依存性である。

【0005】また、エリスロポエチンの存在下に維持するヒト巨核芽球性白血病細胞株UT-7（Komatsu et al., Cancer Res. 51:341, 1991）もTF-1細胞と同様にGM-CSFやIL-3にも依存性である。一方、GM-CSFおよびIL-3依存性を消失したUT-7/Epo細胞の樹立が報告されている（Komatsu et al., Blood 82:456, 1993）。しかしながら、UT-7/Epo細胞のGM-CSFおよびIL-3依存性の消失が、GM-CSF、IL-3共通のシグナル伝達タンパク質である受容体β鎖の量的減少に起因しているものである以上（Komatsu et al., 前出）、親株であるUT-7細胞のSCF依存性（Auffray et al., Exp. Hematol. 22:417, 1994）を有している可能性は否定できない。

【0006】また、近年、エリスロポエチン特異的なシグナル伝達機構の解明が精力的に行われ、それにかかわる多くの物質が同定されている。しかし、これらの研究は前述したTF-1細胞（Hanazono et al., Int. J. Hematol. 59:80, 1994）や、B細胞系であるBa/F3細胞にエリスロポエチン受容体を人為的に発現させた細胞（例えば、Showers et al., Blood 80:3070, 1992）を用いて行われており、これらの細胞はエリスロポエチンのみならず、GM-CSFやIL-3にも依存性を示す。このような様々なサイトカインに反応する細胞を用いた場合には、エリスロポエチンで刺激した場合であっても他のサイトカインのシグナル伝達経路を流用している可能性が否定できない。

【0007】したがって、エリスロポエチン存在下に樹立され、継代を重ねてもエリスロポエチンのみに依存して増殖し、かつエリスロポエチンに特異的なシグナル伝達タンパク質/遺伝子の発見にも使用できるような細胞株は得られていなかった。

【0008】

【発明が解決すべき課題】このような状況の中で、生理的なエリスロポエチン標的細胞である赤芽球の性質を残し、かつエリスロポエチンのみに特異的に依存して増殖し、エリスロポエチン活性のスクリーニングに用いることができ、またエリスロポエチンに特異的なシグナル伝達タンパク質/遺伝子の発見にも使用できる細胞株の樹立が求められている。

【0009】本発明の目的はエリスロポエチン活性のスクリーニングに使用できるエリスロポエチン依存性細胞株を提供することにある。

【0010】本発明はさらに、該細胞株を用いてエリスロポエチン活性を測定するインビトロスクリーニング法を提供することも目的とする。

【0011】

【課題を解決するための手段】本発明者らは、上記目的を達成すべく鋭意研究した結果、エリスロポエチンの生理的標的細胞である赤芽球が白血病化した細胞の中か

ら、かかる細胞株を樹立することに成功し、本発明を完成した。

【0012】すなわち、本発明はエリスロポエチンに特異的に依存して増殖するヒト細胞株を提供する。

【0013】さらに、本発明はエリスロポエチン活性を有すると推察される化合物を上記細胞株に添加して細胞増殖または分化誘導を試験することからなるエリスロポエチン活性のインビトロスクリーニング法を提供する。

【0014】本発明の細胞株は、例えば初期赤芽球白血病 (early erythroblastic leukemia) 患者の骨髓などから採取した細胞を用いて樹立することができる。本発明では特に、骨髓に芽球が76.2%認められた患者の骨髓芽球を採取した。この細胞はEpo添加でコロニーを形成した。さらにこの芽球をFCS (ウシ胎児血清) およびEpo添加したIMDM (イスコーブ改変ダルベッコ培地) にて培養したところ継代可能となり、安定した細胞株を樹立することができた。このようにして樹立された細胞株はAS-E2と命名され、生命工学工業技術研究所特許微生物寄託センターに受託番号FIRM:BP-4819で寄託されている (1994年10月5日寄託)。

【0015】AS-E2はパーオキシダーゼ反応 (PO) が陰性であり、巨赤芽球様変化を認めた (過ヨウ素酸シッフ試薬: PAS陽性)。

【0016】また、細胞株AS-E2の表面抗原を検討したところ、CD71、CD36、グリコホリンA (GPA)、CD38、CD19などを発現していた。

【0017】AS-E2の各種サイトカインに対する反応性を試験したところ、Epoとともにインキュベートしたときのみ顕著に刺激され、AS-E2がEpoに特異的に反応して増殖することを示した。さらに、AS-E2に対するEpoの反応性および抗Epo抗体による増殖阻害を試験するために顕微鏡下で細胞数を数え、またトリパンブルーで生細胞数をカウントしたところ、Epo添加条件にのみ顕著な細胞増殖が認められ、また抗Epo抗体の添加によってこのような増殖効果は抑制されることが明らかとなった。

【0018】また、放射標識Epoの結合実験よりAS-E2におけるEpo受容体の発現が確認された。さらに得られたデータからScatchard分析を用いてEpo受容体の性質を解析した結果、AS-E2が発現するEpo受容体は低親和性型のみであった。

【0019】最近、EpoはEpo受容体と結合し、これによってJakファミリーチロシンキナーゼの1種であるJak2がリン酸化され、さらにこのリン酸化によってJak2自身がキナーゼ活性をもって次の分子をリン酸化するという増殖シグナルの伝達経路が提唱されている (Ihle et al., TIBS 19:222, 1994)。この仮説の正当性を検討するために、AS-E2をEpo非存在下で24時間培養し、次いでEpoを添加した後、細胞を

可溶化し、抗Jak2抗体または抗Epo受容体抗体で免疫沈降を行った。沈降物をSDS-PAGEに付した後、PVDF膜に転写し、抗リン酸化チロシン抗体でチロシンリン酸化タンパク質を検出した結果、AS-E2はEpoに反応し、かつEpoシグナル伝達系であるEpo受容体およびJak2のチロシンリン酸化が認められた。これは、上記の増殖シグナル伝達経路が正しいことを示唆する結果といえる。

【0020】増殖シグナルとは別に、同じ受容体から発信される赤血球特異的な細胞分化のシグナルの存在が示唆されているが、これについては現在ほとんど解明されていない。ただ、Epo受容体から赤血球特異的な一連の転写因子群を活性化するシグナルが伝達され、これら転写因子によって制御されている下流の赤血球特異的な遺伝子群 (ヘム合成酵素遺伝子やグロビン遺伝子) の転写が活性化され、最終的にヘモグロビン産生細胞に分化すると考えられている (Yamamoto M. 実験医学11:1026, 1993)。この特異的な転写因子であるGATA-1、GATA-2のmRNAの発現をノーザンブロットにより試験したところ、AS-E2ではGATA-1およびGATA-2のいずれもが発現していることが確認された。

【0021】このようなAS-E2の各種特徴から、AS-E2がEpoのみに特異的に依存して増殖する細胞株であることが示された。

【0022】したがって、本発明の細胞株は各種組換え体EpoまたはEpo活性を有すると推察される低分子化合物などのEpo活性を簡単かつ確実にインビトロでスクリーニングするのに有用である。このようなスクリーニングは細胞増殖または分化誘導を試験することによって実施できる。例えば、Epo活性を有すると推察される化合物と本発明の細胞株とを混合してインキュベーションし、一定時間後の細胞増殖を³T-チミジンの取り込み試験、またはMTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) アッセイ法 (J. Immun. Methods 65:55, 1983参照) で測定するか、あるいは生細胞数を測定することなどによって実施することができる。また、この試験法によってEpo濃度を測定することも可能である。さらに、本発明の細胞株を、初期赤芽球白血病の病態解析や治療モデルとして使用することができる。さらには、上述したように、本発明の細胞株を、エリスロポエチンに特異的なシグナル伝達タンパク質/遺伝子を解明するための実験にも使用することができる。

【0023】以下に本発明を実施例によりさらに詳しく説明するが、本発明の範囲はこれに限定されるものではない。

【0024】
【実施例】

実施例1: AS-E2の樹立および維持

初期赤芽球白血病患者から骨髓液を採取した。この患者は初診時骨髓に芽球〔光顕PO(−)、PAS(−)、CD11b(−)、CD13(+)、CD33(+)、CD10(−)、CD19(−)、グリコホリンA(+)、染色体:46,XY〕が76.2%認められた。この患者の骨髓芽球はEpo添加でコロニーを形成した。次いでこの芽球を、Epo(2u/ml)および20% FCS添加IMDM中で培養したところ安定して継代可能となり、細胞株として樹立された。この細胞株をAS-E2と命名した。

【0025】AS-E2の形態および表面抗原は以下の通りである。

表1

	陽性率 (%)
CD2	0.2
CD10	0.2
CD19	6.5
CD25	0
CD34	0.7
CD36	98.8
CD38	14.1
CD41	0.7
CD71	99.1
HLA-DR	1.1
グリコホリンA	43.1

表から明らかなように、AS-E2はCD71、CD36、グリコホリンA(GPA)、CD38、CD19などを発現していた。

【0028】実施例2:各種サイトカインに対する反応性

実施例1で樹立した細胞株AS-E2の各種サイトカイ

ヒトエリスロポエチン(hEPO):10u/ml
 ヒトインターロイキン-1 α (hIL-1 α):10ng/ml
 ヒトインターロイキン-1 β (hIL-1 β):10ng/ml
 ヒトインターロイキン2(hIL-2):10ng/ml
 ヒトインターロイキン3(hIL-3):100ng/ml
 ヒトインターロイキン4(hIL-4):10ng/ml
 ヒトインターロイキン6(hIL-6):10ng/ml
 ヒトインターロイキン7(hIL-7):10ng/ml
 ヒトインターロイキン8(hIL-8):1000ng/ml
 ヒトインターロイキン11(hIL-11):10ng/ml
 ヒト顆粒球コロニー刺激因子(hG-CSF):100u/ml
 ヒト顆粒球マクロファージコロニー刺激因子(hGM-CSF):10ng/ml
 ヒト白血病阻害因子(hLIF):10ng/ml
 ヒト赤血球分化因子(hEDF):10ng/ml
 ヒト幹細胞因子(hSCF):10ng/ml
 ヒトトランスフォーミング増殖因子- β (hTGF- β):100ng/ml
 ヒト塩基性繊維芽細胞増殖因子(hbFGF):10ng/ml
 ヒト肝細胞増殖因子(hHGF):500ng/ml
 ヒトオンコスタチンM:100ng/ml

AS-E2の形態

AS-E2は顕微鏡で観察したところ、径約30 μ 程度であり、空胞を有している。POは陰性であり、PASは陽性であった。

AS-E2の表面抗原の解析

本実施例で樹立した細胞株AS-E2の表面抗原の発現を、各種ヒト抗原に対するモノクローナル抗体のパネルを用いて、フローサイトメーターによる蛍光抗体法(Fried et al., Flow Cytometry, Boca Raton, CRC Press: 59-78, 1989)により検討した。

【0026】得られた結果を以下の表1に示す。

【0027】

ンへの反応性を試験した。

【0029】AS-E2 5,000細胞/ウェルに各種サイトカインを添加し、5% CO₂、95% 空気(湿度100%)、37℃で3日間培養した。使用したサイトカインの種類および濃度は以下の通りである:

ヒト上皮増殖因子 (hEGF) : 1000 ng/ml

培養後に Cell Titer 96™ Aqueous Non-Radioactive Cell Proliferation Assay Kit (Promega 社製) を用いて 490 nm における吸光度を測定することにより細胞数を測定した。細胞を含まないブランク、およびサイトカインを含まない対照を同時に試験した。得られた結果を図 1 に示す。図から明らかなように、AS-E2 は EPO とともにインキュベートしたときのみ顕著に増殖が刺激され、その他のサイトカインによつては刺激されなかった。

実施例 3 : EPO 反応性および抗 EPO 抗体による増殖阻害

実施例 1 で樹立した細胞株 AS-E2 の EPO 反応性 (増殖活性) および抗 EPO 抗体による増殖活性を試験した。

【0030】 AS-E2 細胞 1×10^5 細胞/ml を用いて、(1) EPO を添加しないで培養、(2) EPO 2 u/ml を添加して培養、および (3) EPO 2 u/ml と抗 EPO 抗体 ($\alpha \text{ EPO} : 1/100$ に希釈して使用) を添加して培養し、経時的 (2、4、6、8 日) に細胞数を顕微鏡下でカウントした。得られた結果を図 2 に示す。図から明らかなように、EPO 添加した細胞は EPO 添加しない細胞と比較して細胞数の顕著な増加が観察された。また、抗 EPO 抗体を同時に添加した場合には、この増殖活性が阻害された。

【0031】 次いで、上記 3 群の細胞をトリパンブルーで染色して生細胞数をカウントした結果を図 3 に示す。図から明らかなように、EPO 添加しない細胞、および EPO と抗 EPO 抗体を添加した細胞では生細胞数が経時的に減少していたが、EPO 添加した細胞では細胞数としてカウントされたほとんどが生細胞数としてもカウントされた。

実施例 4 : EPO 受容体の発現の確認

AS-E2 細胞上で EPO 受容体が発現していることを以下の方法で確認した。

【0032】 AS-E2 細胞を 24 時間 EPO 非存在下で培養し、 1×10^6 個の細胞に対して $^{125} \text{I-EPO}$ (Amersham 社製) を $0.06 \sim 3 \text{ nM}$ 添加して 15°C 、3 時間の条件で結合アッセイを行った。非特異的結合は、 $^{125} \text{I-EPO}$ の 400 倍量の非標識 EPO 存在下で測定した。なお、結合アッセイ培地の組成は、2% BSA、0.2% NaN_3 を含む IMDM 培地である。得られた結合を図 4 に示す。これより、AS-E2 細胞上に EPO 受容体が発現していることが確認された。

【0033】 次いで結合アッセイから得られたデータから Scatchard 分析を行って EPO 受容体の解離定数 (K_d 値) と受容体数とを求めたところ、AS-E2 細胞が発現している EPO 受容体が低親和性型 1 種類

のみであることが示された。

実施例 5 : Jak2、EPO 受容体のリン酸化

AS-E2 における Jak2 および EPO 受容体のリン酸化を以下の方法で試験して EPO のシグナル伝達経路を検討した。

【0034】 AS-E2 を EPO 非存在下で 24 時間培養し、次いで EPO (0 または 10 u/ml) を添加して 5 分間反応させた後、1% NP-40 (ナカライテスク株式会社製)、10% グリセロール、1 mM PMSF (和光純薬工業株式会社製)、 0.1 mg/ml ロイペプチン (和光純薬工業株式会社製)、 $5 \mu \text{M}$ ペプスタチン A (和光純薬工業株式会社製)、1 mM EDTA、2 mM オルトバナジウム酸ナトリウム (SIGMA 社製) を含むトリス緩衝塩溶液で細胞を可溶化した。これを用いて抗 Jak2 抗体または抗 EPO 受容体抗体 (いずれも UPSTATE BIOTECHNOLOGY, INC. (USA) 社製) で免疫沈降を行った。沈降物を SDS-PAGE に付した後、PVDF 膜に転写し、抗リン酸化チロシン抗体 (和光純薬株式会社製) でチロシンリン酸化タンパク質を検出した。得られた結果を図 5 (A : 抗 Jak2 抗体、B : 抗 EPO 受容体抗体、また、A、B いずれにおいても、レーン 1 は EPO 非添加の場合を、レーン 2 は EPO 添加した場合を表す) に示す。図から明らかなように、AS-E2 は EPO に反応し、かつ EPO シグナル伝達系である Jak2 および EPO 受容体のチロシンリン酸化が認められた。

実施例 6 : GATA-1 および GATA-2 の mRNA の発現

AS-E2 における GATA-1 および GATA-2 の mRNA の発現を以下のノーザンブロット分析により検討した。

【0035】 本発明の細胞株 AS-E2、ならびに対照として K562 細胞株 (慢性骨髄性白血病急性転化細胞株) および HL-60 細胞株 (急性白血病細胞株) から、ISOGEN (ニッポンジーン社製) を用いて、全 RNA をキット添付の処方に従って調製した。次いで、ノーザンブロット分析 (Molecular Cloning: A Laboratory Manual, Sambrook et al., Cold Spring Harbor Laboratory Press, 1989) を行った。プローブの標識は Random Primer Labelling Kit (Takara 社製) を用いて行った。すなわち、PVC ベクターの EcoRI 部位に挿入された GATA-1 (Zon et al., Proc. Natl. Acad. Sci. USA 88:10638-10641, 1991) と GATA-2 (Nagai et al., Blood 84:1074-1084, 1994) をコーディングするインサートを切り出し、キット添付の処方に従って標識プローブを作製した。

【0036】 前述の各細胞からの全 RNA を 1 レーン当

たり30 μ g使用し、アガロースゲル電気泳動を行った後、ハイブリダイゼーション・トランスファー・メンブレンとしてHybond-N (Amersham社製)を使用し、キャピラリー法によりプロットした。ハイブリダイゼーションは50%ホルムアミド、5 \times SSPE、2 \times Denhardt's solution、0.1% SDSの組成よりなる溶液を用いて42 $^{\circ}$ Cで一晩実施した。ハイブリダイゼーション後、メンブレンを2 \times SSC、0.1% SDSで室温にて4回洗浄し、さらにBAS (Fujii社製)にてオートラジオ

グラフィを行い、画像解析をした。その結果を図6 (A: GATA-1、B: GATA-2、また、A、Bいずれにもレーン1はAS-E2、レーン2はK562、レーン3はHL-60を表す)に示す。図から明らかなように、GATA-1はAS-E2とK562に明瞭なバンドとして検出され、またGATA-2はAS-E2、K562、HL-60のいずれの細胞株でも認められた。

【0037】

【発明の効果】本発明によってエリスロポエチンのみに特異的に依存して増殖し、エリスロポエチン活性のスクリーニングに用い得る細胞株の樹立が達成された。本発明の細胞株を用いてエリスロポエチン活性を有すると推定される化合物のエリスロポエチン活性を測定するインビトロスクリーニング法が可能となった。このインビトロスクリーニングは本発明の細胞株の細胞増殖または分化誘導を試験することにより実施できる。また、この試験法によってEpo濃度を測定することも可能である。さらに、本発明の細胞株を、初期赤芽球白血病の病態解析や治療モデルとして使用することができる。さらに

は、本発明の細胞株を用いて、エリスロポエチンに特異的なシグナル伝達タンパク質/遺伝子を解明するための実験を行うことができる。

【0038】これらの点から本発明のエリスロポエチン依存性ヒト細胞株の利用価値は極めて大きい。

【図面の簡単な説明】

【図1】AS-E2細胞に対する各種サイトカインの反応性を示す図である。

【図2】AS-E2のEpo反応性および抗Epo抗体による増殖活性を示すための、顕微鏡で観察した経時的な細胞数を示す図である。(●)はEpo 2u/mlを添加した細胞、(△)はEpoを添加しない細胞、(□)はEpo 2u/mlと抗Epo抗体(α Epo: 1/100希釈)を添加した細胞を表す。

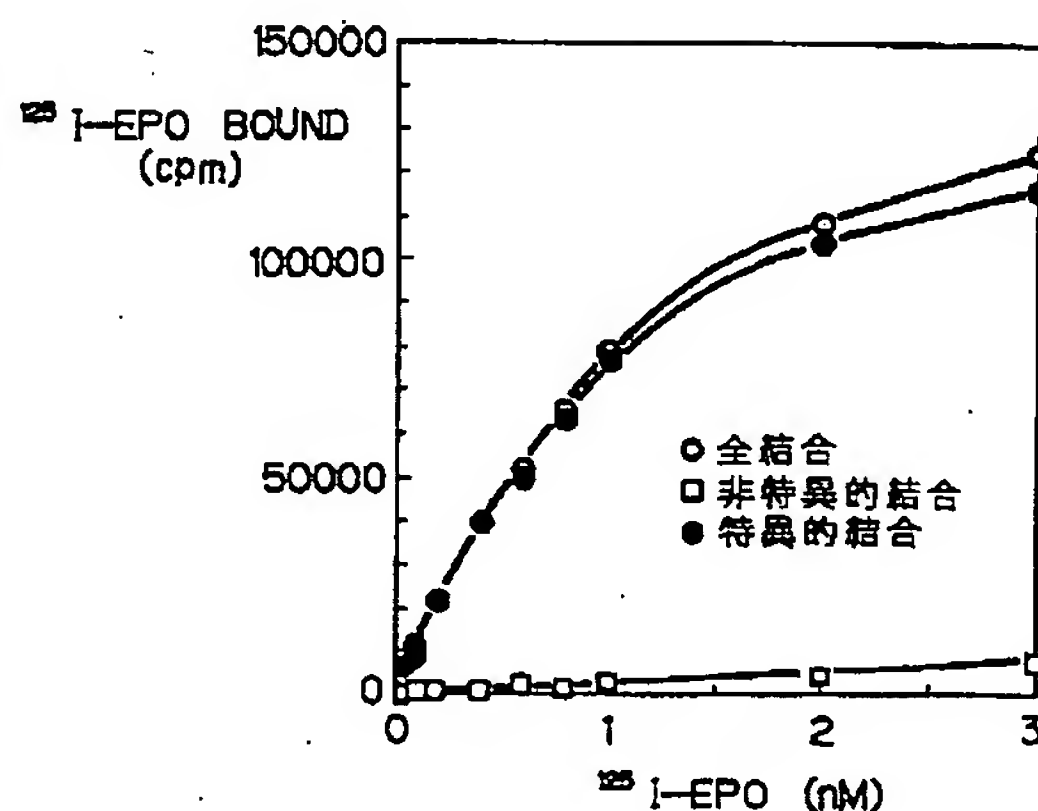
【図3】AS-E2のEpo反応性および抗Epo抗体による増殖活性を示すための、トリパンブルー染色した経時的な生細胞数を示す図である。(●)はEpo 2u/mlを添加した細胞、(△)はEpoを添加しない細胞、(□)はEpo 2u/mlと抗Epo抗体(α Epo: 1/100希釈)を添加した細胞を表す。

【図4】 125 I-Epoを用いるAS-E2の結合アッセイを示す図である。(○)は全結合活性、(□)は非特異的結合活性、(●)は特異的結合活性を表す。

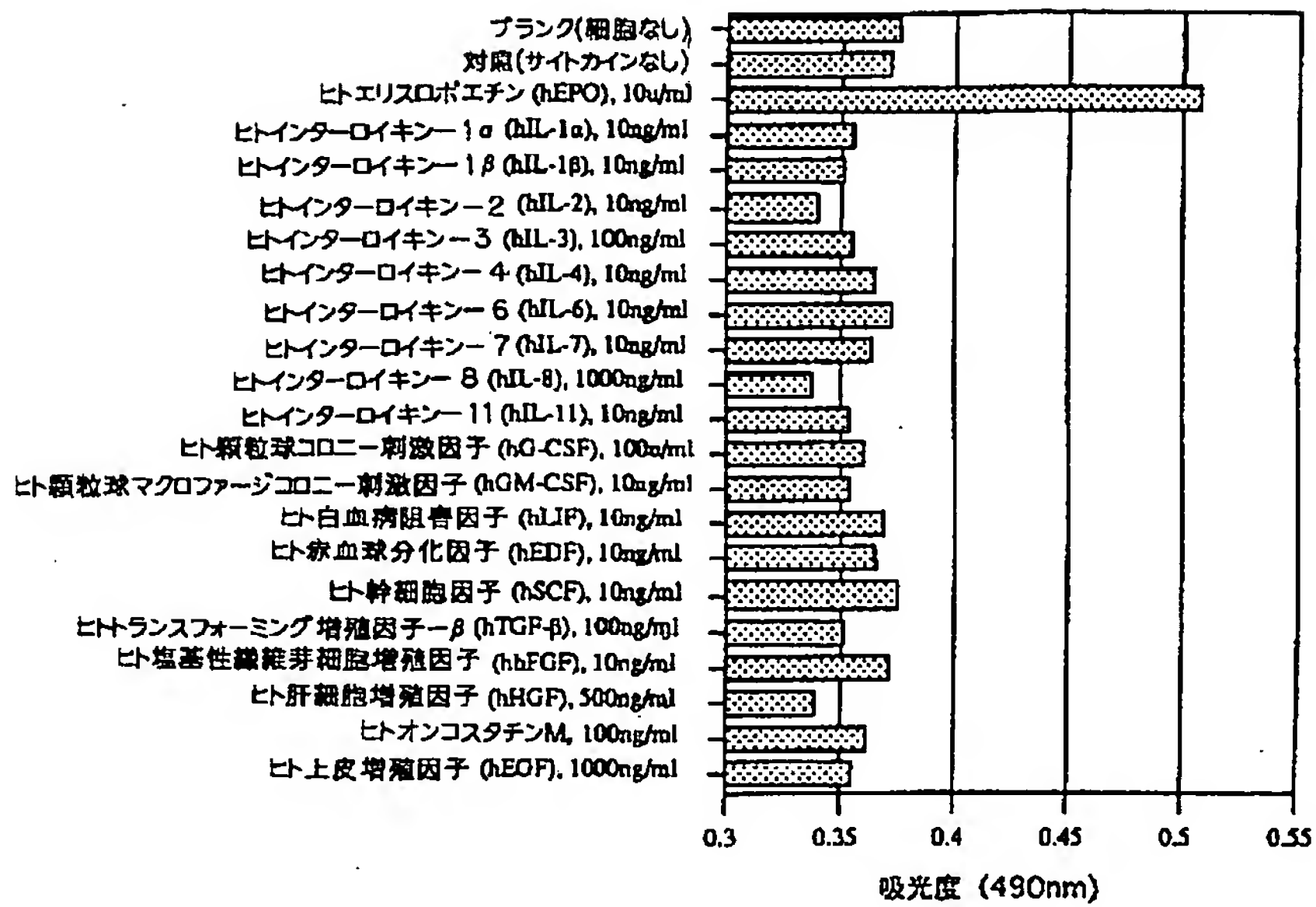
【図5】AS-E2におけるJak2およびEpo受容体のリン酸化を示す図(電気泳動の写真)である。図中、Aは抗Jak2抗体(レーン1はEpo非添加;レーン2はEpo添加)、Bは抗Epo受容体抗体(レーン1はEpo非添加;レーン2はEpo添加)との免疫沈降物をSDS-PAGEの電気泳動に付し、PVDF膜に転写し、抗リン酸化チロシン抗体でチロシンリン酸化タンパク質を検出した図を表す。

【図6】AS-E2、K562、HL-60細胞株におけるGATA-1、GATA-2のmRNAの発現を示す図(電気泳動の写真)である。図中、AはGATA-1(レーン1はAS-E2;レーン2はK562;レーン3はHL-60)、BはGATA-2(レーン1はAS-E2;レーン2はK562;レーン3はHL-60)をプローブとして用いたノーザンブロットの結果を表す。

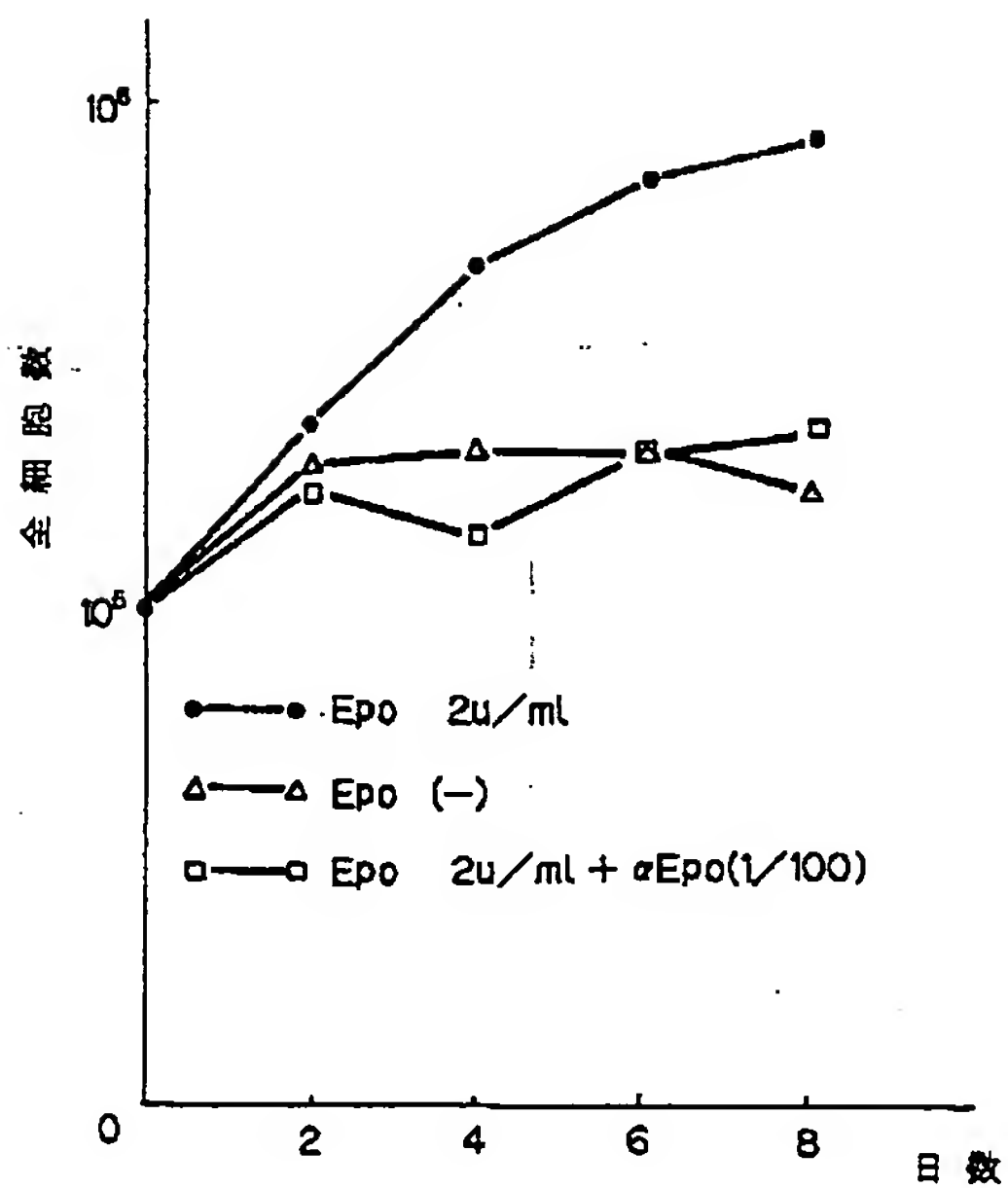
【図4】



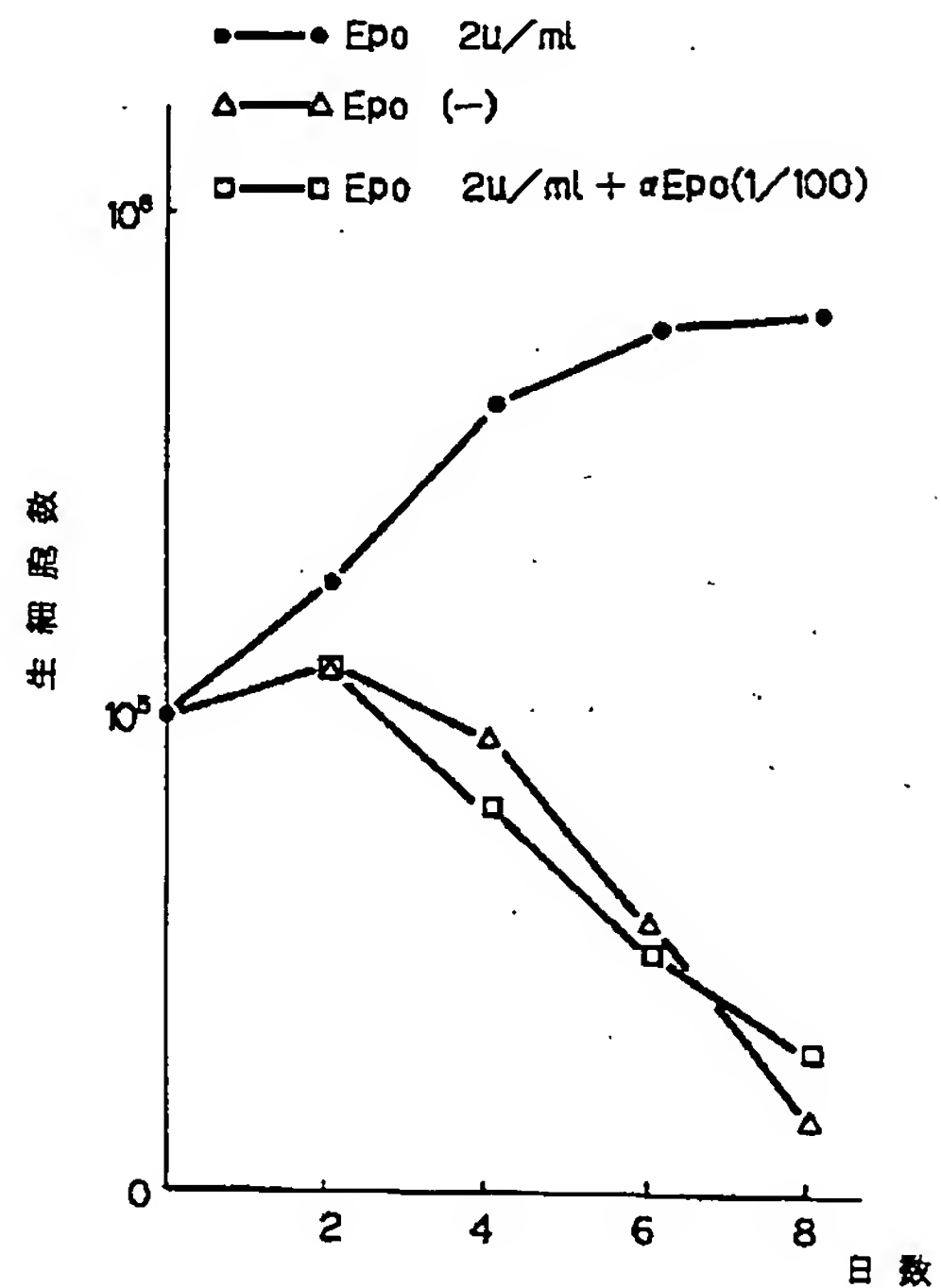
【図1】



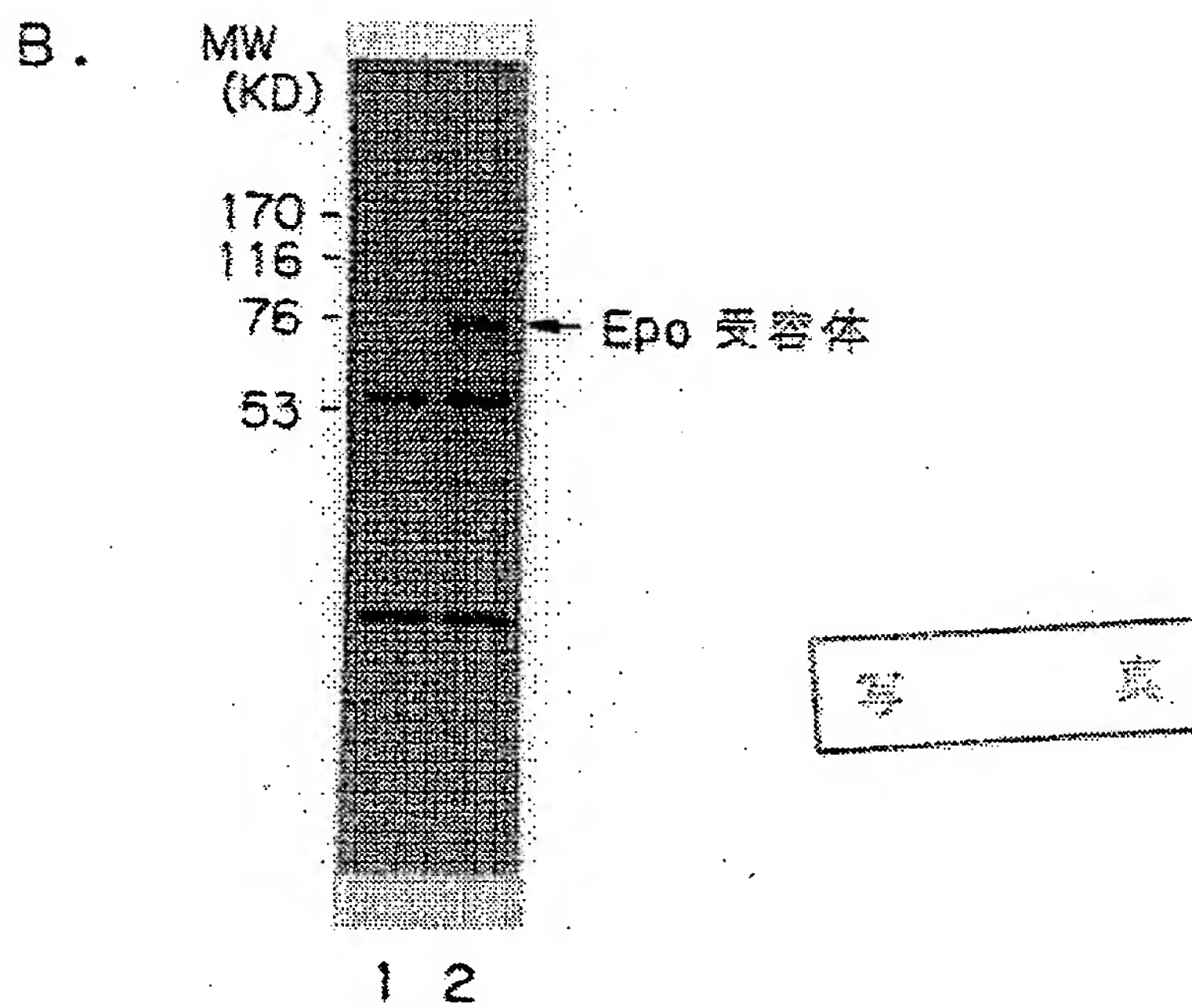
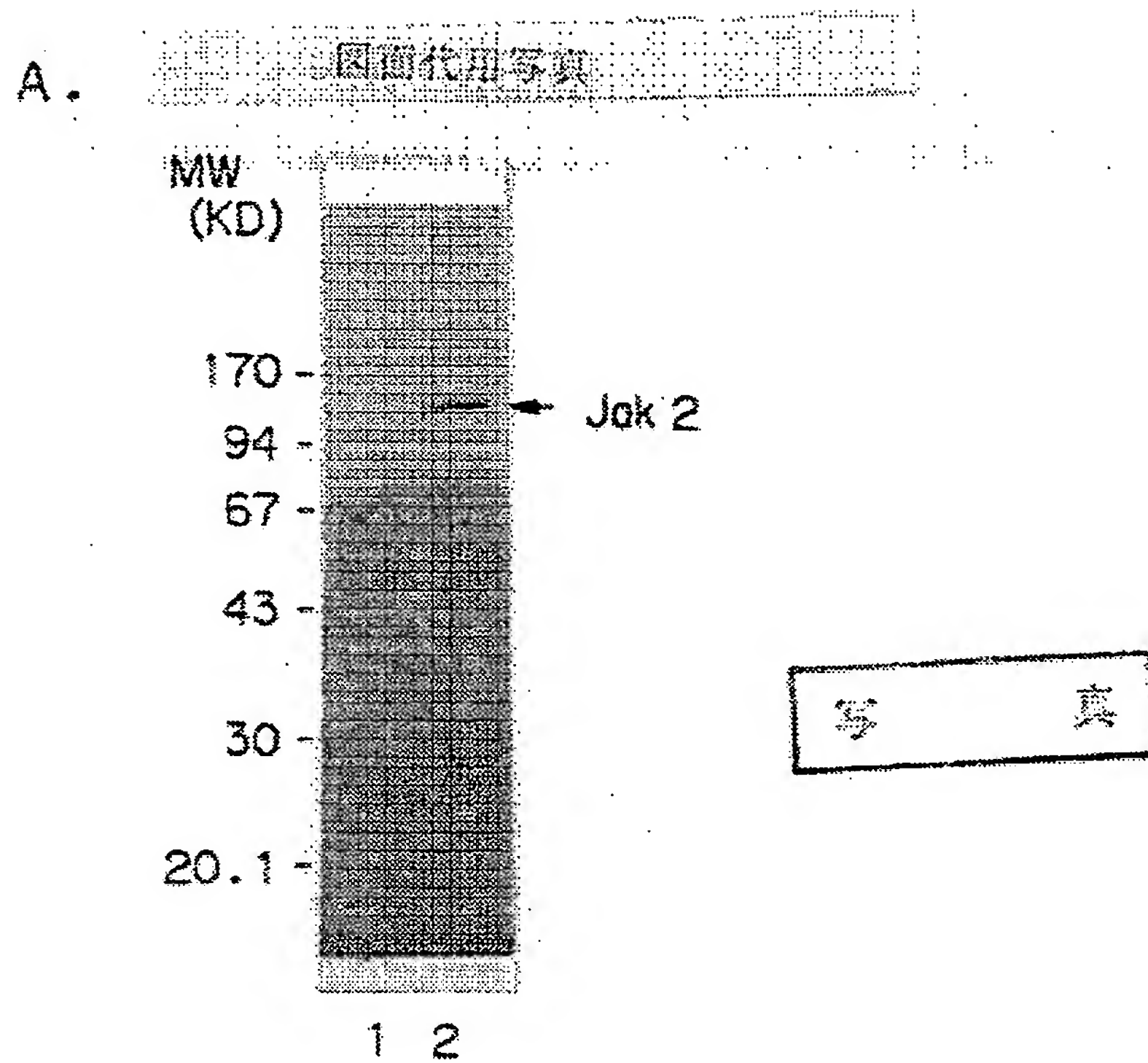
【図2】



【図3】

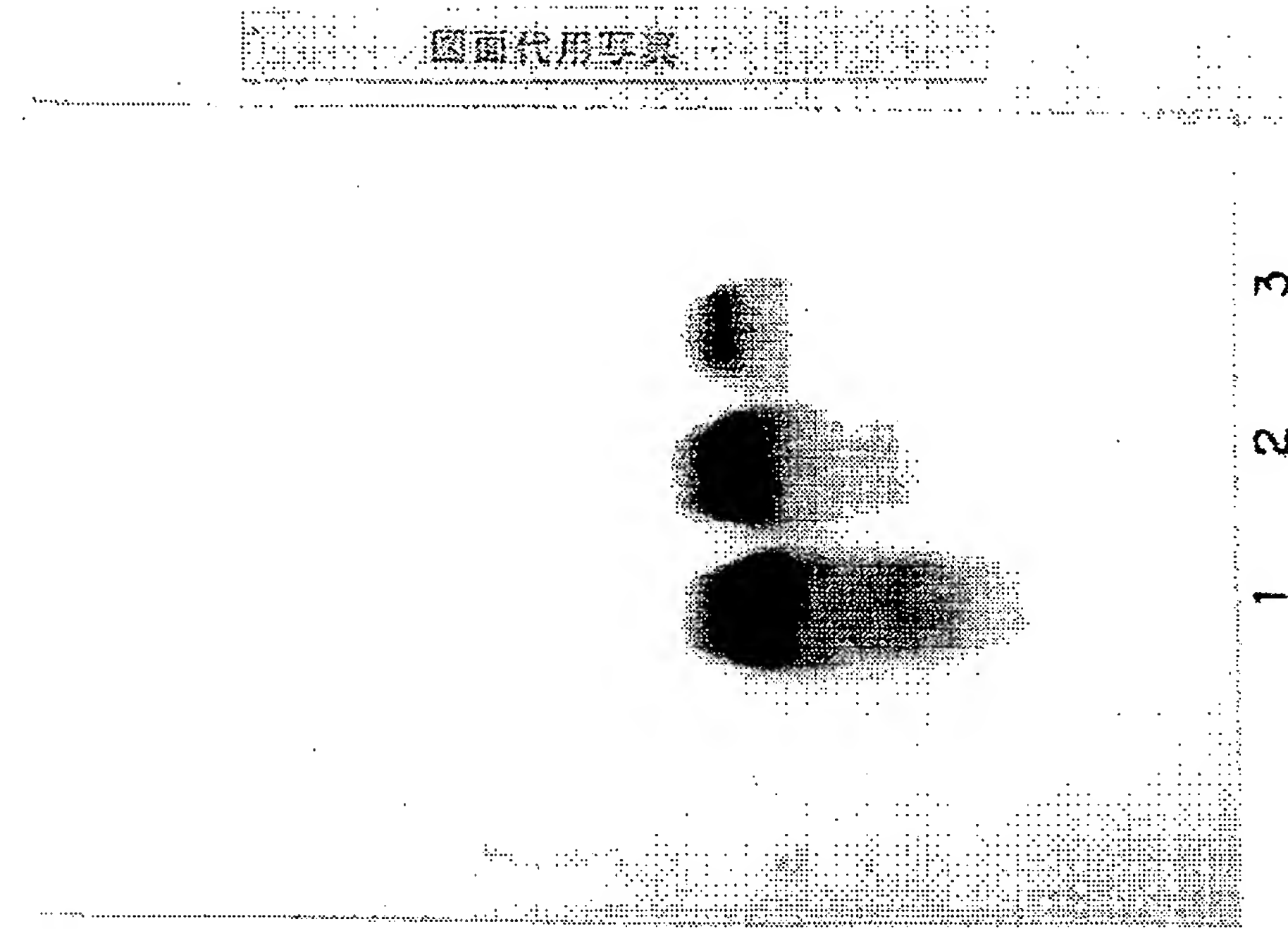


【図5】

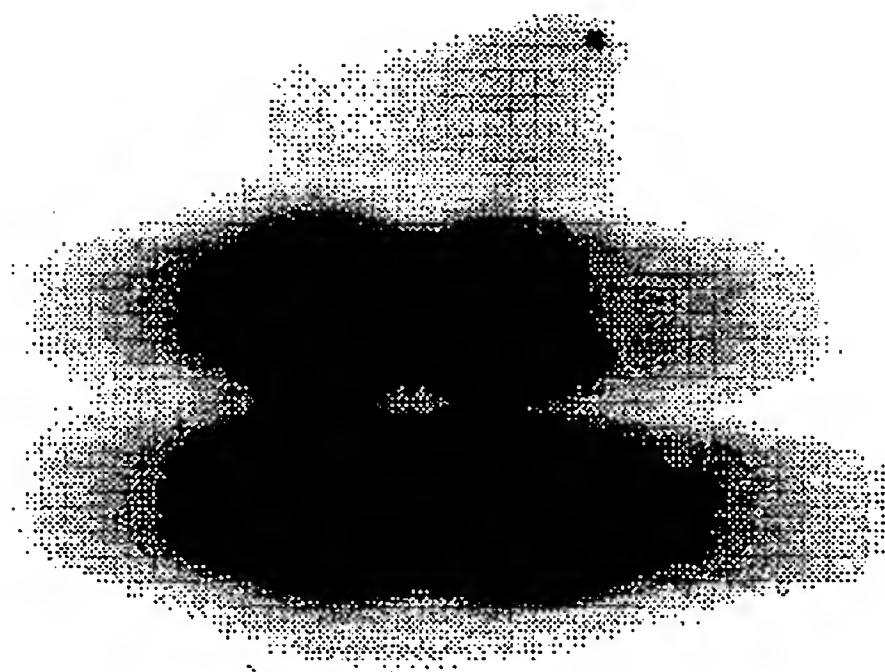


【図6】

図面代用写真



B.



A.

写真

フロントページの続き

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(51) Int. Cl. ⁶				
G 0 1 N 33/53	V			
	K			
/(C 1 2 N 5/06				
C 1 2 R 1:91)				
			A 6 1 K 37/02	A C C
			(C 1 2 N 5/00	E
			C 1 2 R 1:91)	

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